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The Patent Office

Cardiff Road
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1. Your reference — 9381 GB

2. Patent application number *(The Patent Office will fill in this part)* — 11 JUL 2003 — **0316294.8**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

   Polytherics Limited
   90 Fetter Lane
   London EC4A 1JP

   Patents ADP number *(if you know it)* 08589939001

   If the applicant is a corporate body, give the country/state of its incorporation — UK

4. Title of the invention — Conjugated Biological Molecules and their Preparation

5. Name of your agent *(if you have one)* — Abel & Imray

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

   20 Red Lion Street
   London
   WC1R 4PQ
   United Kingdom

   Patents ADP number *(if you know it)* — 174001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
   a) any applicant named in part 3 is not an inventor, or
   b) there is an inventor who is not named as an applicant, or
   c) any named applicant is a corporate body.
   See note (d))

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Description	31
Claim *(s)*	4
Abstract	0
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Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	1
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Abel & Imray.

Date 11 July 2003

12. Name and daytime telephone number of person to contact in the United Kingdom — Sue Scott 020 7242 9984

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Conjugated Biological Molecules and their Preparation

This invention relates to conjugated biological molecules and their preparation from novel chemically functionalised derivatives of polymers such as polyethylene glycol.

Many therapeutically active molecules do not possess the properties required to achieve efficacy in clinical medical use. For example, therapeutically active proteins and polypeptides are now being discovered and produced by the biopharmaceutical industry and by genetic engineering. Although there are currently at least 80 protein based medicines marketed in the United States with at least 350 more protein based medicines undergoing clinical trails (Harris J, Chess R: Effect of Pegylation on pharmaceuticals. Nature Review Drug Discovery, 2003, 2, 214-221), most native proteins do not make good medicines because upon administration to patients there are several inherent drawbacks that include: (1) proteins are digested by many endo-and exopeptidases present in blood or tissue, (2) many proteins are immunogenic to some extent and (3) proteins can be rapidly excreted by kidney ultrafiltration. Other molecules used as active therapeutic agents in medicines that are systemically toxic or lack optimal bioavailability and pharmacokinetics include low molecular weight molecules where an effective dose is limited by toxicity. Such molecules are routinely used to treat inflammation and conditions due to malignancies, infection and autoimmune disease.

Water soluble, synthetic polymers, particularly polyalkylene glycols, are used to conjugate therapeutically active molecules such as proteins. These therapeutic conjugates have

been shown to favourably alter pharmacokinetics by prolonging circulation time and decreasing clearance rates, decrease systemic toxicity, and in several cases, to display increased clinical efficacy. This process of covalently conjugating polyethylene glycol, PEG, to proteins is commonly known as "PEGylation" however many different polymers have been examined as conjugating polymers.

Many polymer reagents for conjugation comprise conjugating chemical functionality that is hydrolytically unstable. Examples of hydrolytically unstable polymeric conjugating reagents are active esters that include, for example, polyalkylene oxide-N-succinimide carbonates (Zalipsky US patent No. 5,122,614). These reagents have relatively short half lives in aqueous media, that includes blood or plasma. This results in the need to add large stoichiometric excesses of the conjugating polymer reagent. The hydrolytic stability of the reagent is important because the requirement to add stoichiometric excesses for protein conjugation requires significant effort and cost to purify the polymer-protein conjugate from the reaction mixture. Furthermore, these hydrolytically unstable reagents tend to undergo preferentially, reaction with amine chemical functionality in the protein, particularly to the ε-amine of lysine residues. Since most proteins of interest have more than one lysine residue, and frequently many lysine residues, then conjugation tends to be non-specific in that it occurs at many residue sites on the protein. It is possible to purify the conjugating reaction mixture to isolate proteins conjugated to one polymer molecule, however it is not possible, to isolate at a reasonable cost, polymer-protein conjugates that are all conjugated to the same amine group on the protein. Non-specific conjugation frequently results in impaired protein function. For example antibodies and

antibody fragments with random poly(alkylene oxide) attachment via lysine residues result in modified antibodies (or modified antibody fragments) able to bind target antigen with reduced affinity, avidity or specificity. Additionally, amine specific polymer conjugating reagents require conjugating reaction conditions that must be selected to ensure that the amines on the protein are not protonated. These conditions require moderately high pH media (8-10), this allows the amine moieties to be reactive enough for reaction with the polymer conjugating reagent. High pH conditions are frequently deleterious to the protein causing structural changes and denaturation. These processes result in impairment of protein function. Amine specific polymer conjugation reagents tend to bind to accessible amine sites on the protein. These reagents can be termed kinetic reagents. They are labile and undergo a reaction with the most assessable amino nucleophilic sites on the protein. Amine specific polymer conjugating reagents that conjugate by amine acylation result in the loss of positive charge on the amine group of the amino acid residue on the protein that would normally be present under physiological conditions for the unconjugated protein. These features of amine specific polymer conjugating reagents often leads to partial impairment of the function of the protein. Other conjugating functional groups incorporated in polymers for conjugation to protein and that are amine specific and frequently hydrolytically labile include isocyanate (WO 94/04193) and carbonates (WO 90/13540).

Particularly relevant for optimised efficacy and to ensure dose to dose consistency is to make certain that the number of conjugated polymer molecules per protein is the same and that each polymer molecule is specifically covalently conjugated to the same amino acid residue in each protein

molecule. Non-specific conjugation at sites along a protein molecule results in a distribution of conjugation products and frequently, unconjugated protein, to give a complex mixture that is difficult; tedious, and expensive to purify.

Thiol specific polymer conjugating reagents for proteins have been developed to address the limitations for the propensity of the conjugating reagent to undergo hydrolysis that is competitive with conjugation to the protein, non-specific polymer conjugation at different amino acid residues in the protein, and the need for high pH conjugating reaction conditions. Thiol specific polymer conjugating reagents can be utilised at pH values close to neutrality where the amine functional moieties on the amino acid residues of the protein are protonated and thus cannot effectively compete in the conjugation reaction with the polymer conjugating reagent. Thiol specific polymer conjugating reagents that are relatively more hydrolytically stable than are the aforementioned amine specific reagents can be utilised at a lower stoichiometric excess thus reducing the cost during purification of the polymer-protein conjugate. Conjugating functional moieties that are broadly selective for thiol groups include iodoacetamide, maleiimide (WO 92/16221), vinylsulfone (WO 95/13312 and WO 95/34326), vinyl pyridines (WO 88/05433), and acrylate and methacrylate esters (WO 99/01469). These thiol selective conjugating moieties yield a single thioether conjugating bond between the polymer.

Most proteins do not have free sulfhydrals because these sulfhydrals undergo rearrangement and scrambling reactions with the disulfide bridges within the protein resulting in impaired protein function. For proteins that do have free sulfhydrals, these sulfhydrals are frequently critical for protein function. Typically in a protein, the number of

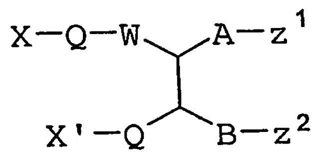
sulfhydral moieties is less than the number of amine moieties (e.g. lysine or histadine). Since conjugation to a protein can be made to be specific at thiol groups and since proteins do not typically have free thiol groups, there are examples of site-specific modification of protein by mutagenesis to introduce thiol sites for PEG attachment. However such modifications increase costs significantly. The introduced free sulhydral can have the similar limitations as mentioned heretofore in the engineered protein for protein scrambling and protein dimerisation. Also the process of mutagensis and production of the modified protein from bacterial sources frequently causes the free sulfhydral to be bound in a disulfide bond with glutathione, for example. Interleukin-2, for example, has been modified by mutagenesis to replace a threonine residue by a cysteine to allow site specific attachment of PEG. [Goodson RJ, Katre NV; Bio/Technology (1990) 8, 343-346].

It is known in the art that conjugating parameters have to be optimally matched with the therapeutically active molecule of interest in terms of polymer morphology, molecular weight characteristics, chemical functionality. Although the polymer protein conjugate, can display many favourable and necessary properties needed for safe, effective medical use, the effect of polymer conjugation on the activity and stability of the protein is of vital importance for performance. Conjugation variables related to the location and amount of conjugation and polymer characteristics must be optimally correlated with biological and physicochemical properties.

We have now found a series of novel reagents which can be used *inter alia* to conjugate with both sulphur atoms derived from two cysteine residues in a protein to give novel thioether conjugates. The technology provides clear

advantages over known techniques for conjugating polymers to proteins.

The present invention provides a compound of the general formula



(I)

in which one of X and X′ represents a polymer, and the other represents a hydrogen atom;

each Q independently represents a linking group;

W represents an electron-withdrawing moiety or a moiety preparable by reduction of an electron-withdrawing moiety; or, if X′ represents a polymer, X-Q-W- together may represent an electron withdrawing group; and in addition, if X represents a polymer, X′ and electron withdrawing group W together with the interjacent atoms may form a ring;

each of Z^1 and Z^2 independently represents a group derived from a biological molecule, each of which is linked to A and B via a nucleophilic moiety; or Z^1 and Z^2 together represent a single group derived from a biological molecule which is linked to A and B via two nucleophilic moieties;

A is a C_{1-5} alkylene or alkenylene chain; and

B is a bond or a C_{1-4} alkylene or alkenylene chain.

A polymer X or X′ may for example be a polyalkylene glycol, a polyvinylpyrrolidone, a polyacrylate, for example polyacryloyl morpholine, a polyoxazoline, a polyvinylalcohol, a polyacrylamide or polymethacrylamide, for example

polycarboxymethacrylamide, or a HPMA copolymer. Additionally X or X′ may be a polymer that is susceptible to enzymatic or hydrolytic degradation. Such polymers, for example, include polyesters, polyacetals, poly(ortho esters), polycarbonates, poly(imino carbonates), and polyamides, such as poly(amino acids). A polymer X or X′ may be a homopolymer, random copolymer or a structurally defined copolymer such as a block copolymer. For example X or X′ may be a block copolymer derived from poly(alkylene oxide) and either a polyester, polyacetal, poly(ortho ester), or a poly(amino acid). Polyfunctional polymers that may be used include copolymers of divinylether-maleic anhydride and styrene-maleic anhydride. Naturally occurring polymers may also be used, for example polysaccharides such as chitin, dextran, dextrin, chitosan, starch, cellulose, glycogen, and derivatives thereof. Polymers such as polyglutamic acid may also be used.

If the polymer is a polyalkylene glycol, this is preferably one containing C_2 and/or C_3 units, and is especially a polyethylene glycol. A polymer, particularly a polyalkylene glycol, may contain a single linear chain, or it may have branched morphology composed of many chains either small or large. Substituted polyalkylene glycols, for example methoxypolyethylene glycol, may be used. In a preferred embodiment of the invention, a single-chain polyethylene glycol is initiated by a methoxy group and is connected at the other end of the chain to the linker group Q.

The polymer X or X′ may optionally be derivatised or functionalised in any desired way. For example, polymers with two or more chemical moieties for conjugation that are the subject of this invention may be used to create conjugates of two or more linked bioactive molecules.

Reactive groups may be linked at the polymer terminus or end group, or along the polymer chain through pendent linkers; in such case, the polymer is for example a polyacrylamide, polymethacrylamide, polyacrylate, polymethacrylate, or a maleic anhydride copolymer. Multimeric conjugates that contain more than one biological molecule, typically a biologically active polypeptide or drug can result in synergistic and additive benefits. If desired, the polymer may be coupled to a solid support using conventional methods.

The optimum molecular weight of the polymer will of course depend upon the intended application. Preferably, the number average molecular weight is in the range of from 500g/mole to around 75,000g/mole. When the compound of the general formula I is intended to leave the circulation and penetrate tissue, for example for use in the treatment of inflammation caused by malignancy, infection or autoimmune disease, it may be advantageous to use a lower molecular weight polymer in the range 2000-30,000g/mole. For applications where the compound of the general formula I is intended to remain in circulation it may be advantageous to use a higher molecular weight polymer, for example in the range of 20,000 - 75,000g/mole.

The polymer to be used should be selected so the conjugate is soluble in the solvent medium for its intended use. For biological applications, particularly for diagnostic applications and therapeutic applications for clinical therapeutical administration to a mammal, the conjugate will be soluble in aqueous media. Many proteins such as enzymes have utility in industry, for example to catalyze chemical reactions. For conjugates intended for use in such applications, it may be necessary that the conjugate be soluble in either or both aqueous and organic media. The

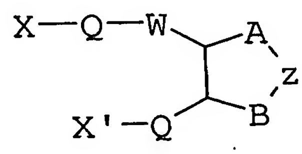
polymer should not impair the intended function of the biological molecule(s).

A linking group Q may for example be a direct bond, an alkylene group (preferably a C_{1-10} alkylene group), or an optionally-substituted aryl or heteroaryl group, any of which may be terminated or interrupted by one or more oxygen atoms, sulphur atoms, -NR groups (in which R has the meaning given below), keto groups, -O-CO- groups and/or -CO-O- groups. Suitable aryl groups include phenyl and naphthyl groups, while suitable heteroaryl groups include pyridine, pyrrole, furan, pyran, imidazole, pyrazole, oxazole, pyridazine, primidine and purine.

Substituents which may be present on an optionally substituted aryl or heteroaryl group include for example one or more of the same or different substituents selected from -CN, $-NO_2$, $-CO_2R$, -COH, $-CH_2OH$, -COR, -OR, -OCOR, $-OCO_2R$, -SR, -SOR, $-SO_2R$, -NHCOR, -NRCOR, $-NHCO_2R$, $-NR'CO_2R$, -NO, -NHOH, -NR'OH, -C=N-NHCOR, -C=N-NR'COR, $-N^+R_3$, $-N^+H_3$, $-N^+HR_2$, $-N^+H_2R$, halogen, for example fluorine or chlorine, -C≡CR, $-C=CR_2$ and -C=CHR, in which each R or R' independently represents a hydrogen atom or an alkyl (preferably C_{1-6} alkyl) or an aryl (preferably phenyl) group. The presence of electron withdrawing substituents is especially preferred.

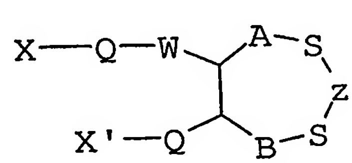
W may for example represent a keto or aldehyde group CO, an ester group -O-CO- or a sulphone group $-SO_2-$, or a group obtained by reduction of such a group, e.g. a CH.OH group, an ether group CH.OR, an ester group CH.O.C(O)R, an amine group $CH.NH_2$, CH.NHR or $CH.NR_2$, or an amide CH.NHC(O)R or $CH.N(C(O)R)_2$. If X-Q-W- together represent an electron withdrawing group, this group may for example be a cyano group.

In the following portion of this specification, Z^1 and Z^2 will be referred to collectively as Z. It is a preferred embodiment of the invention that Z^1 and Z^2 together should represent a single biological molecule:

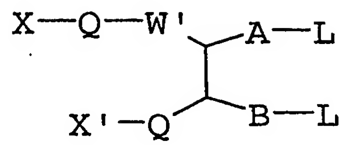


Z may be derived from any desired biological molecule, for example a protein. The protein may for example be a polypeptide, antibody, enzyme, cytokine, chemokine or receptor. Constrained or cyclic polypeptides, which are usually cyclised through a disulphide bridge, and epitopes, may also be used. Specific examples of suitable biological molecules are listed below.

Preferably a nucleophilic moiety linking A or B to the group(s) Z is derived from a thiol group or amine group. Two thiol groups may be generated by partial reduction of a natural or engineered disulphide (cysteine) bridge. Amine groups may for example be lysine residues. Where Z^1 and Z^2 together form a single biological molecule which is linked to the groups A and B via two thiol groups, the compound of the formula I has the formula



The invention also provides a process for the preparation of a compound of the general formula I, which comprises reacting either (i) a compound of the general formula



(II)

in which one of X and X′ represents a polymer and the other represents a hydrogen atom;

Q represents a linking group;

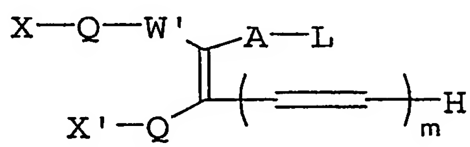
W′ represents an electron-withdrawing group, for example a keto group, an ester group -O-CO- or a sulphone group $-SO_2-$; or, if X′ represents a polymer, X-Q-W′ together may represent an electron withdrawing group;

A represents a C_{1-5} alkylene or alkenylene chain;

B represents a bond or a C_{1-4} alkylene or alkenylene chain; and

each L independently represents a leaving group;

or (ii) a compound of the general formula



(III)

in which X, X′, Q, W′, A and L have the meanings given for the general formula II, and in addition if X represents a polymer, X′ and electron-withdrawing group W′ together with the interjacent atoms may form a ring, and m represents an integer 1 to 4; with a compound of the general formula

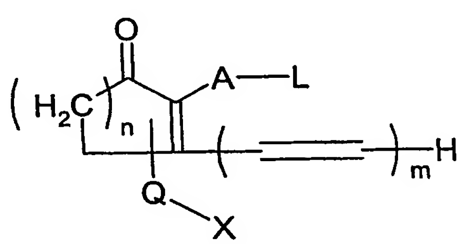
$$Z(Nu)_2 \qquad (IV)$$

in which Z has the meaning given above and each Nu independently represents a nucleophilic group, for example a thiol or an amine group. If Z^1 and Z^2 are separate molecules,

the reaction takes place in two successive steps with successive molecules Z^1Nu and Z^2Nu.

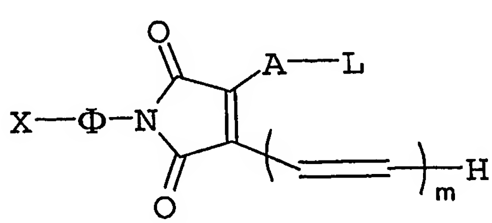
The or each leaving group L may for example represent $-SR$, $-SO_2R$, $-OSO_2R$, $-N^+R_3$, $-N^+HR_2$, $-N^+H_2R$, halogen, or $-O\emptyset$, in which R has the meaning given above, and Ø represents a substituted aryl, especially phenyl, group, containing at least one electron withdrawing substituent, for example $-CN$, $-NO_2$, $-CO_2R$, $-COH$, $-CH_2OH$, $-COR$, $-OR$, $-OCOR$, $-OCO_2R$, $-SR$, $-SOR$, $-SO_2R$, $-NHCOR$, $-NRCOR$, $-NHCO_2R$, $-NR'CO_2R$, $-NO$, $-NHOH$, $-NR'OH$, $-C{=}N{-}NHCOR$, $-C{=}N{-}NR'COR$, $-N^+R_3$, $-N^+HR_2$, $-N^+H_2R$, halogen, especially chlorine or, especially, fluorine, $-C{\equiv}CR$, $-C{=}CR_2$ and $-C{=}CHR$, in which R and R' have the meanings given above.

Typical structures in which W' and X' together form a ring include



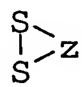
(IIIa)

in which n is an integer from 1 to 4, and



(IIIb)

A compound of the general formula (IV) in which each Nu is a thiol group may be prepared by partial reduction of a protein contain a cysteine link, i.e.



(V)

Suitably, the process according to the invention is carried out by partially reducing a disulfide bond derived from two cysteine amino acids in the protein *in situ* following which the reduced product reacts with the compound of formula (II) or (III). Disulfides can be reduced, for example, with dithiothreitiol, mercaptoethanol, or tris-carboxyethylphosphine using conventional methods. The process may be carried out in a solvent or solvent mixture in which all reactants are soluble. The biological molecule containing nucleophilic groups (e.g. protein) may be allowed to react directly with the compound of the general formula II or III in an aqueous reaction medium. This reaction medium may also be buffered, depending on the pH requirements of the nucleophile. The optimum pH for the reaction is generally between about 5.5 and about 8 and preferably about 7.4. Reaction temperatures between 3-37 °C are generally suitable: proteins and other biological molecules may decompose or denature impairing function if the conjugation reaction is conducted at a temperature where these processes may occur. Reactions conducted in organic media (for example THF, ethyl acetate, acetone) are typically conducted at temperatures up to ambient, for example temperatures below 0°C.

A protein can contain one or a multiplicity of disulfide bridges. Reduction to give free sulfhyral moieties can be conducted to reduce one or a multiplicity of disulfide bridges in a protein. Depending on the extent of disulfide

reduction and the stoichiometry of the polymeric conjugation reagent that is used, it is possible to conjugate one or a multiplicity of polymer molecules to the protein. Alternatively the source of the thiol groups can be from cysteines or thiols not originally derived from a disulfide bridge. If the source of the thiol groups is a disulfide bridge, this may be intrachanin or interchain.

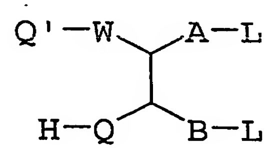
The biological molecule can be effectively conjugated with the reagents of the present invention using a stoichiometric equivalent or a slight excess of reagent, unlike many prior art reagents.

The compounds of formulae (II) and (III) are novel, and accordingly the invention further provides these compounds *per se*. These novel reagents provide a breakthrough in conjugate technology, the chemical functional moiety on the polymer comprising a cross-functionalised, latently cross-conjugated, bis-alkylating moiety that is selective for two nucleophiles, particularly two thiols derived from a natural disulfide bond in proteins.

The immediate product of the process according to the invention is a compound of the general formula I in which W is an electron-withdrawing group. Such compounds have utility in themselves; because the process of the invention is reversible under suitable conditions, compounds of formula (I) in which W is an electron-withdrawing moiety have utility in applications where release of the free protein is required, for example in direct clinical applications. An electron-withdrawing moiety W may, however, be reduced to give a moiety which prevents release of the protein, and such compounds have utility in many clinical, industrial and diagnostic applications.

Thus, for example, a moiety W containing a keto group may be reduced to a moiety W containing a CH(OH) group; an ether group CH.OR may be obtained by the reaction of a hydroxy group with an etherifying agent; an ester group CH.O.C(O)R may be obtained by the reaction of a hydroxy group with an acylating agent; an amine group $CH.NH_2$, CH.NHR or $CH.NR_2$ may be prepared from a ketone or aldehyde by reductive amination); or an amide CH.NHC(O)R or $CH.N(C(O)R)_2$ may be formed by acylation of an amine). A group X-Q-W- which is a cyano group may be reduced to an amine group.

A compound of the general formula (II) in which X represents a polymer may be prepared by reacting a compound of the general formula

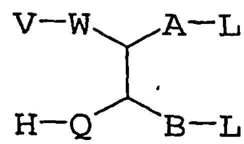


(VI)

in which Q', W, A, B and L have the meanings given above, with a polymer of the general formula

$$X - V \qquad (VII)$$

in which X represents a polymer; Q' and V being groups selected such that the compounds of (VI) and (VII) will react together to give the desired compound of the formula (II). Alternatively, a compound of the formula



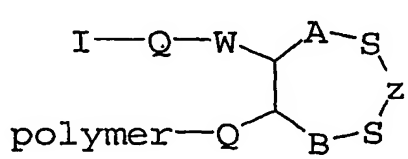
(VIII)

may be reacted with a polymer of the general formula

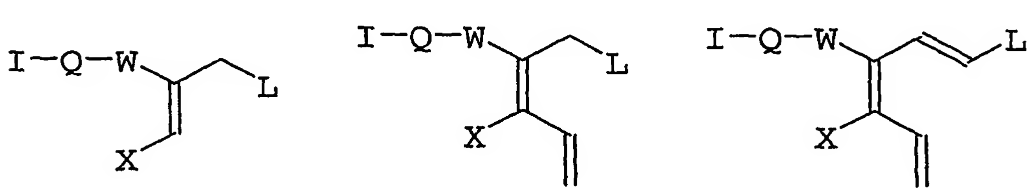
X - Q′ (IX)

A compound of the general formula (III) may be prepared by base mediated elimination of one leaving group L from a compound of the general formula (II).

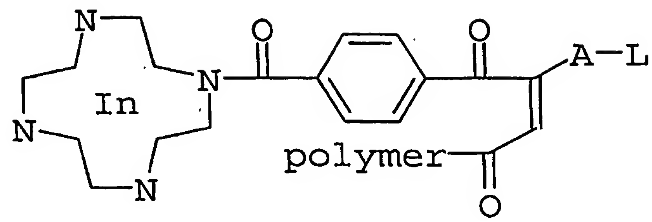
Compounds of the general formula I may include an imaging agent, for example a radio nucleotide, to enable tracking of the compound *in vivo*. Suitably the radio nucleotide or imaging agent I may be bound through the group W, to give, for example, compounds of the type



which may for example be prepared from reagents of the type



for example



The compounds of the general formula I have a number of applications. They may for example be used for direct clinical application to a patient, and accordingly, the present invention further provides a pharmaceutical composition comprising a compound of the general formula I together with a pharmaceutically acceptable carrier. The invention further provides a compound of the general formula I for use as a medicament, and a method of treating a patient which comprises administering a pharmaceutically-effective amount of a compound of the formula I or a pharmaceutical composition according to the invention to the patient. Any desired pharmaceutical effect, for example enzyme replacement, toxin removal, anti-inflammatory, anti-infective, immunomodulatory, vaccination or anti-cancer, may be obtained by suitable choice of biological molecule.

The compounds of the general formula I may also be used in non-clinical applications. For example, many physiologically active compounds such as enzymes are able to catalyse reactions in organic solvents, and compounds of the general formula I may be used in such applications. Further, compounds of the general formula I may be used as diagnostic tools.

The following gives some specific biological molecules which may have utility in the present invention, depending upon the desired application. Enzymes include carbohydrate-specific

enzymes, proteolytic enzymes and the like. Enzymes of interest, for both industrial (organic based reactions) and biological applications in general and therapeutic applications in particular include the oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases disclosed by US 4,179,337. Specific enzymes of interest include asparaginase, arginase, adenosine deaminase, superoxide dismutase, catalase, chymotrypsin, lipase, uricase, bilirubin osidase, glucose oxidase, glucuronidase, galactosidase, glucocerbrosidase, glucuronidase, glutaminase

The biologically active molecules used in compounds of the general formula I of the present invention include for example factor 8, insulin, ACTH, glucagen, somatostatin, somatotropins, thymosin, parathyroid hormone, pigmentary hormones, somatomedins, erythropoietin, luteinizing hormone, hypothalamic releasing factors, antidiuretic hormones, prolactin, interleukins, interferons, colony stimulating factors, hemoglobin, cytokines, antibodies, chorionicgonadotropin, follicle-stimulating hormone, thyroid stimulating hormone and tissue plasminogen activator.

Certain of the above proteins such as the interleukins, interferons and colony stimulating factors also exist in non-glycosilated form, usually the result of preparation by recombinant protein techniques. The non-glycosilated versions may be used in the present invention.

Other proteins of interest are allergen proteins disclosed by Dreborg et all Crit. Rev. Therap. Drug Carrier Syst. (1990) 6 315 365 as having reduced allergenicity when conjugated with a polymer such as poly(alkylene oxide) and consequently are suitable for use as tolerance inducers. Among the allergens

disclosed are Ragweed antigen E, honeybee venom, mite allergen and the like.

Glycopolypeptides such as immunoglobulins, ovalbumin, lipase, glucocerebrosidase, lectins, tissue plasminogen activator and glycosilated inerleukins, interferons and colony stimulating factors are of interest, as are immunoglobulins such as IgG, IgE, IgM, IgA, IgD and fragments thereof.

Of particular interest are antibodies and antibody fragments which are used in clinical medicine for diagnostic and therapeutic purposes. The antibody may used alone or may be covalently conjugated ("loaded") with another atom or molecule such as a radioisotope or a cytotoxic/antiinfective drug. Epitopes may be used for vaccination to produce an immunogenic polymer - protein conjugate.

A key feature of the process of the invention is that an α-methylene leaving group and a double bond are cross-conjugated with an electron withdrawing function that serves as a Michael activating moiety. If the leaving group is prone to elimination in the cross-functional reagent rather than to direct displacement and the electron-withdrawing group is a suitable activating moiety for the Michael reaction then sequential intramolecular bis-alkylation can occur by consecutive Michael and retro Michael reactions. The leaving moiety serves to mask a latent conjugated double bond that is not exposed until after the first alkylation has occurred and bis-alkylation results from sequential and interactive Michael and retro-Michael reactions as described in J. Am. Chem. Soc. 1979, 101, 3098-3110 and J. Am. Chem. Soc. 1988, 110, 5211-5212.). The electron withdrawing group and the leaving group are optimally selected so bis-alkylation can occur by sequential Michael and retro-Michael reactions.

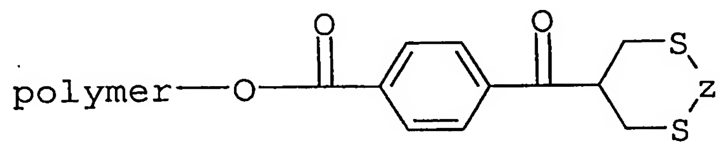
It is also possible to prepare cross-functional alkylating agents with additional multiple bonds conjugated to the double bond or between the leaving group and the electron withdrawing group as described in J. Am. Chem. Soc. 1988, 110, 5211-5212.

Since the cross-functionalised bis-alkylating reagents of the type mentioned above undergo alkylation that is controlled by Michael-retro Michael equilibria and since it is possible to partially reduce a number from one to a greater than one disulfide bonds in a protein in a controlled fashion significantly retaining tertiary structure, it is then possible to have bis-alkylation occur across the two sulfhydrals from cysteins of a given disulfide bond. Such a sequence of reactions results in the reannealling of the disulfide bridge with the bis-alkylating reagent.

The thiol ether bonds formed upon conjugation to give a compound of the formula I are, in general, hydrolytically stable in aqueous solution. The reagents themselves are also hydrolytically stable. In this context, a compound is regarded as being hydrolytically stable if it does not undergo substantial degradation at physiological pH and temperature up to 45°C. Degradation of less than 50% under these conditions over an eight hour period is considered insubstantial.

It will be appreciated that this invention allows the production of polymer reagents that possess cross-functionalised bis-alkylating functionality at either the termini or on pendent chains along the main chain of a polymer.

Some examples of novel conjugates according to the invention include the following:



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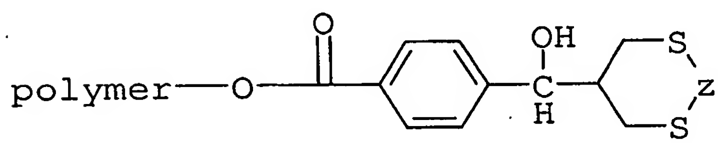
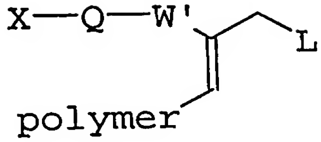
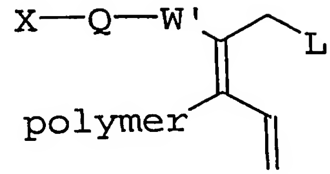
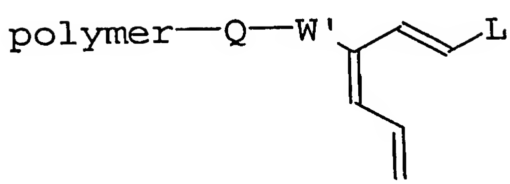
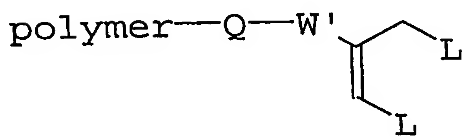


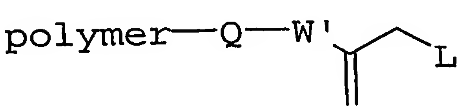
Some examples of novel reagents according to the invention include the following:

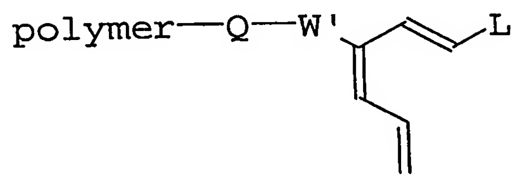


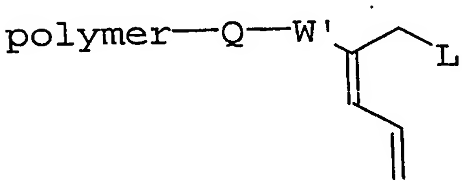












The following Examples illustrate the invention.

Example 1. Synthesis of polymer conjugating reagent.

p-Nitro-3-piperidinopropriophenone hydrochloride: $C_{14}H_{19}ClN_2O_3$

To a single-neck 250 ml round-bottom flask was added *p*-nitroacetophenone (16.5 g), paraformaldehyde (4.5 g), piperidine hydrochloride (12.1 g), absolute ethanol (100 mL) and a magnetic stir bar. To the stirred heterogeneous mixture was added hydrochloric acid (37 wt% in water, 1 mL) and the solution was heated to reflux under nitrogen. After a 1-2 h period more paraformaldehyde (3.0 g) was added. The solution was allowed to reflux for approximately 18 h during which

time further paraformaldehyde was added (3.0 g). After allowing the reaction solution to cool a crystalline solid settled that would not dissolve upon further refluxing. The solid was isolated by filtration and recrystallised using very hot methanol to afford large yellow crystals (10.9 g). ^1H NMR (DMSO-d_6) δ 1.34-1.50 (m, 1H), 1.64-1.79 (m, 2H), 1.79-1.94 (m, 4H), 2.89-3.05 (m, 2H), 3.41 (q, 2H), 3.51-3.54 (m, 2H), 3.82 (t, 2H), 8.26 (s, 1H), 8.29 (s, 1H), 8.41 (s, 1H), 8.44 (s, 1H).

2,2-Bis(p-tolylthiolmethyl)-p-nitroacetophenone: $C_{24}H_{23}NO_3S_2$

To a 100 ml single-neck round-bottom flask was added *p*-nitro-3-piperidinopropiophenone hydrochloride (10.0 g), 4-methylbenzenethiol (8.2 g), formaldehyde (37% w/w aq. solution, 10 ml, excess), methanol (40 ml) and a magnetic stir bar. The stirred heterogeneous mixture was heated until a yellow homogeneous solution formed (a couple of minutes at 50 - 60°C). Five drops of piperidine were then added and the reaction solution heated to reflux. Within 15 min the reaction became heterogeneous due to the presence of some white/yellow solid and after 2 h this solid became a strong orange in colour. The refluxing was stopped after this time and the reaction was allowed to cool overnight to room temperature. The reaction mixture was then heated under reflux again with additional formaldehyde (37% w/w aq. solution, 10 ml, excess). After refluxing for approximately 30 min an orange oil was visible and no solid. The oil would settle to the bottom of the flask when the stirring was stopped. After a further 7 h of refluxing, the mixture was allowed to cool overnight, which resulted in the settled oil crystallising. The crystalline solid was isolated and purified by recrystallisation from very hot methanol with several drops of acetone added to afford yellow crystals

(10.0 g). ^{1}H NMR (DMSO-d_6) δ 2.31 (s, 6H), 3.31-3.33 (m, 4H), 3.97 (quintet, 1H), 7.14 (q, 8H), 7.80 (d, 2H), 8.24 (d, 2H).

2,2-Bis(p-tolylsulfonylmethyl)-p-nitroacetophenone: $C_{24}H_{23}NO_7S_2$

In a 250 ml round-bottom flask a suspension of 2,2-bis(*p*-tolylthiolmethyl)-*p*-nitroacetophenone (2.5 g) and Oxone (18.4 g) was stirred in a 1:1 methanol:water (100 ml) for 16 h. This afforded a white solid suspension, to which was added chloroform (100 ml) and the resulting organic phase was isolated using a separating funnel to leave a white solid suspension within the aqueous phase. Additional water was added to the aqueous phase until a homogeneous solution formed, which was then washed again with chloroform (100 ml). The organic phases were combined, washed with brine (50 mL x 2), dried with magnesium sulfate and the solvent removed affording an off-white crude solid product after drying in vacuo (2.5 g). The product was recrystallised from acetone to give white crystals. ^{1}H NMR ($CDCl_3$) δ 3.43-3.62 (m, 4H), 4.44 (quintet, 1H), 7.35 (d, 4H), 7.68 (d, 4H); 7.88 (d, 2H), 8.22 (d, 2H); analysis calculated for $C_{24}H_{23}NO_7S_2$ (found): C, 57.47 (57.27); H, 4.62 (4.74); N 2.79 (2.58); MS (FAB) m/z 502 ($[M+1]^+$).

2,2-bis[(p-tolylsulfonyl)methyl]-p-aminoacetophenone hydrochloride $C_{24}H_{26}ClNO_5S_2$

To a 100 mL round bottom flask was added 2,2-bis(*p*-tolylsulfonylmethyl)-*p*-nitroacetophenone (2 g), ethanol (25 mL), hydrochloric acid (37 wt.% in water, 8 mL) and a magnetic stir bar. To the resulting heterogeneous mixture was then added tin(II) chloride dihydrate and the mixture heated in an oil bath at 45°C for 2 h. Water was then added to the homogeneous yellow solution that had formed to a point where

it appeared that a precipitation may occur if more water was added. The homogenous solution was allowed to cool to room temperature resulting in a yellow compound crystallising/precipitating out, which was isolated by filtration under vacuum. The isolated product was then mixed with a heated mixture of acetone and methanol (approximately 90:10 v/v). An insoluble solid was isolated by filtration under vacuum and dried to constant mass in a vacuum oven (1.4 g). ^{1}H NMR (DMSO-d_6) δ 2.50 (s, 6H), 3.57-3.73 (overlapping m's, 5H), 6.27 (s, 2H), 6.39 (d, 2H), 6.96 (d, 2H), 7.47 (d, 4H), 7.55 (d, 4H).

Coupling of 2,2-bis[(p-tolylsulfonyl)methyl]-p-aminoacetophenone to α-methoxy-ω-amino PEG

A one neck 100 mL round bottom flask fitted with a dropping funnel and nitrogen line was charged with triphosgene (23 mg), 2,2-bis[(*p*-tolylsulfonyl)methyl]-*p*-aminoacetophenone hydrochloride (125 mg), anhydrous toluene (2.5 mL) and a magnetic stir bar under a nitrogen atmosphere. The dropping funnel was separately charged with anhydrous triethylamine (68 μL) and anhydrous toluene (2.5 mL). An acetone/dry-ice bath was place below the round bottom flask and the contents allowed to cool. The triethylamine solution was then added dropwise to the triphosgene solution under stirring over 5-10 min. The flask and dry-ice bath were allowed to warm to room temperature which took several hours and once at room temperature the reaction mixture was allowed to further stir for about 2 h, still under a nitrogen atmosphere. A solution of O-(2-aminoethyl)-O′-methylpolyethylene glycol 2,000 (490 mg) and anhydrous triethylamine (68 μL) in anhydrous toluene was then added dropwise to the reaction mixture at room temperature. The resulting mixture was allowed to stir at

room temperature overnight (approximately 20 h). The reaction mixture was then opened to the atmosphere and filtered under gravity through a 5 mL disposable syringe with a piece of non-absorbent cotton wool to act as a filter. The homogeneous eluent was transferred to a 100 mL separating funnel and then washed twice with deionised water (30 mL and then 10 mL). The aqueous phases were combined and then washed with diethyl ether (approximately 25 mL). The aqueous phase was then freeze-dried to give an off-white solid product (160 mg). Product was also found in diethyl ether and toluene phases. ^1H NMR ($CDCl_3$) δ 2.5 (s), 3.39 (s), 3.41-3.53 (overlapping m's), 3.53-3.76 (m), 3.82 (t), 4.17 (quintet), 7.35-7.39 (m, 1.39), 7.46 (d), 7.68 (d).

Example 2 - Synthesis of polymer conjugating reagent

p-carboxy-3-piperidinopropriophenone hydrochloride

To a 250 mL single-neck round-bottom flask was added *p*-acetyl benzoic acid (10 g) and piperidine hydrochloride (7.4 g), 100 mL of absolute ethanol and a magnetic stir bar. To the stirred heterogeneous mixture was added concentrated hydrochloric acid (1 mL) and the solution was then heated to reflux under nitrogen. Paraformaldehyde (3.7 g) was added to the flask and refluxing continued for approximately 1.5 h. A homogeneous solution formed to which was added more paraformaldehyde (3.7 g). Heating was continued for approximately 6 h during which time further paraformaldehyde was added (3.7 g.). The reaction solution was allowed to cool to room temperature and left for 1 week. A white solid was isolated by filtration of the cooled reaction mixture. An attempt was made to crystallise the solid after dissolving in very hot methanol. An insoluble product (1.96 g) was isolated by filtration and a second product (0.88 g) crystallised out

of the filtrate. Both products appeared identical by infrared spectroscopy and thin layer chromatography analysis after drying in-vacuo and so were combined to be used in subsequent reactions. ATR-FT-IR 1704, 1691, 1235, 760.

4-[2,2-bis[(p-tolylthio)methyl]acetyl]benzoic acid: $C_{25}H_{24}O_3S_2$

To a 50 mL single-neck round-bottom flask was added *p*-carboxy-3-piperidinopropriophenone hydrochloride (2.5 g), 4-methylbenzenethiol (2.1 g) formaldehyde (37% w/w aq. solution, 2.5 mL), ethanol (10 mL), a magnetic stir bar and piperidine (approximately ten drops). A condenser was then fitted to the flask and the reaction solution heated to reflux. Methanol (5 mL) was then added. After about 2 h, additional formaldehyde (2.5 mL) was added and the heating continued for a further 2 h. The reaction flask was then allowed to cool to room temperature whereupon the reaction solution was diluted with diethyl ether (approximately 150 mL). The resulting organic phase was then washed with water (acidified using 1N hydrochloric acid to pH 2-3; 50 mL x 2), water (50 mL) and brine (75 mL) and then dried over magnesium sulfate. Filtration followed by removal of volatiles on a rotary evaporator afforded a solid residue. The solid was dissolved in a minimum volume of a mixture of predominately methanol and acetone with heating. The homogeneous solution was then placed in a freezer overnight, affording off-white crystals, which were isolated by filtration under vacuum, washed with fresh acetone and then dried to constant mass in a vacuum oven (2.5 g): ^1H NMR ($CDCl_3$) δ 2.38 (s, 6H), 3.16-3.31 (m, 4H), 3.85 (quintet, 1H), 7.15 (d, 4H), 7.18 (d, 4H), 7.64 (d, 2H), 8.07 (d, 2H); analysis calculated for $C_{25}H_{24}O_3S_2$ (found): C, 68.78 (68.84); H, 5.54 (5.77).

4-[2,2-bis[(p-tolylsulfonyl)methyl]acetyl]benzoic acid

$C_{25}H_{24}O_7S_2$

In a 250 mL round-bottom flask a suspension of 4-[2,2-bis[(*p*-tolylthio)methyl]acetyl]benzoic acid (2 g) and Oxone (16.9 g) was stirred in 1:1 v/v methanol:water (100 mL) for 16 h. This afforded a white solid suspension to which was added chloroform (100 mL) and the resulting organic phase was isolated using a separating funnel to leave a white solid suspension with the aqueous phase. To the heterogenous aqueous phase was added additional water until homogenous (approximately 170 mL) and then the aqueous phase was washed with chloroform (75 mL). The organic phases were combined, washed with water (50 mL x 2, acidified with a few drops of 1N hydrochloric acid) and brine (50 mL). The organic phase was dried with magnesium sulfate, filtered and the solvent removed using a rotary evaporator to give an off-white crude solid product (2.2 g). Further purification (on 1 g of product) was performed by recrystallisation from very hot ethyl acetate, acetone and hexane affording 0.6 g of product: ^{1}H NMR ($CDCl_3$) δ 2.51 (s, 6H), 3.49-3.72 (m, 4H), 4.44 (quintet, 1H), 7.40 (d, 4H), 7.73-7.78 (m, 6H), 8.13 (d, 2H); analysis calculated for $C_{25}H_{24}O_7S_2$ (found): C, 59.98 (59.88); H, 4.83 (4.78).

Coupling of 4-[2,2-bis[(p-tolylsulfonyl)methyl]acetyl]benzoic acid to α-methoxy-ω-amino PEG

A one-neck 50 mL schlenk flask was charged with 4-[2,2-bis[(*p*-tolylsulfonyl)-methyl]acetyl]benzoic acid (100 mg) and a magnetic stir bar. The neck part of the flask was sealed with a septum and the flask placed under strong vacuum for approximately 15 min. An argon atmosphere was introduced into the flask and thionyl chloride (1 mL) added by syringe with stirring. The resulting mixture was heated at 50°C for 2 h.

Volatiles were then removed under vacuum to afford a yellow foam. An argon atmosphere was again introduced to the flask and anhydrous dichloromethane (5 mL) was added by syringe to afford a homogeneous solution. Volatiles were then removed again under vacuum. The solvent addition/removal process was repeated once more to afford a white foam. Anhydrous dichloromethane (5 mL) was again added to the schlenk flask to form a homogeneous solution. Separately in a 25 mL round bottom flask fitted with a septum and with a magnetic stir bar, O-(2-aminoethyl)-O′-methylpolyethylene glycol 2,000 (0.2 g), and anhydrous triethylamine (30 μL) were dissolved in anhydrous dichloromethane (5 mL) under an argon atmosphere. The activated sulfone solution was injected into the flask containing the PEG solution in a dropwise fashion, immediately resulting in the evolution of a white gas. The resulting solution was allowed to stir overnight at room temperature whereupon additional triethylamine (28 μL, was added). After a further 1 h, the reaction solution was added dropwise through a glass pipette into rapidly stirring diethyl ether. To achieve precipitation it was necessary to add hexane to the diethyl ether solution and place the flask in an ice bath. The precipitant obtained was isolated by centrifugation and dried in a vacuum oven to constant weight affording an off-white solid product (230 mg). Further purification was achieved by further precipitation by dissolving the product in dichloromethane and adding to chilled diethyl ether under stirring. ^1H NMR ($CDCl_3$) δ 2.51 (s), 3.40 (s), 3.60-3.75 (m), 3.84 (t), 4.36 (quintet), 7.39 (d), 7.68 (d), 7.71 (d), 7.85 (d).

***Reaction of 4-methylbenzenethiol and* α-methoxy-ω-4-[2,2-bis[(p-tolylsulfonyl)methyl]acetyl]benzamide PEG.**

The polymer conjugating reagent, α-methoxy-ω- 4-[2,2-bis[(p-tolylsulfonyl)methyl]acetyl]benzamide PEG (30 mg, 12.1 μmol, 1 eq.) and 4-methylbenzenethiol (3 mg, 24.2 mmol, 2 eq.) were dissolved in deuterated chloroform (approximately 0.75 mL). To the homogeneous solution was then added triethylamine (1.7 μL, 12.1 μmol, 1 eq.). The reaction mixture was stirred and a H-NMR spectrum was obtained. The resulting spectrum indicated that addition of the 4-methylbenzenethiol to the polymer conjugating reagent had occur by a shift in the methyl group signals of 4-methylbenzenethiol observed in the region between 2.0 and 2.5 ppm.

Example 3

Polymer conjugation of Ribonuclease A

To Ribonuclease A (30 mg) in a 15 mL centrifuge tube was added 3 mL of an 8M aqueous urea solution, followed by 2-mercaptoethanol (60 μL). The pH of the resulting solution was adjusted to pH 8.5 using a 10% aqueous solution of methylamine. The reaction solution was then bubbled with nitrogen for approximately 30 min. Still purging with nitrogen the tube was heated at 37°C for 5 h. The reaction mixture was then cooled in an ice-salt water bath and 10 mL of an argon purged chilled solution of 1N HCl:absolute ethanol (1:39 v/v) was added to the reaction solution. A precipitation occurred and the precipitate was isolated by centrifugation and then washed three times with further 10 mL portions of the HCl:absolute ethanol mixture and twice with nitrogen purged chilled diethyl ether (2 x 10 mL). After each washing the precipitate was isolated by centrifugation. The washed precipitate was then dissolved in nitrogen purged de-ionised water and freeze-dried to afford a dry solid. Partial reduction of Ribonuclease A was confirmed and quantitated

using Ellman's Test, which gave 5.9 free thiols per protein molecule.

In an eppendorf, the partially reduced Ribonuclease A (10.9 mg) was dissolved in argon purged pH 8 ammonia solution (500 μL). In a separate eppendorf, the polymer conjugating reagent, α-methoxy-ω- 4-[2,2-bis[(p-tolylsulfonyl)-methyl]acetyl]benzamide PEG (5 mg) was also dissolved in ammonia solution (250 μL) and the resulting solution added to the Ribonuclease A solution. The PEG eppendorf was washed with 250 μL of fresh ammonia solution and this was also added to the main reaction eppendorf. The reaction eppendorf was then closed under argon and heated at 37°C for approximately 24 h and then allowed to cool to room temperature. The cooled reaction solution was then analysed by non-denaturing sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE experiment was consistent with reaction of pegylated 4-[2,2-bis[(p-tolylsulfonyl)methyl]acetyl]-benzoic acid with Ribonuclease A.

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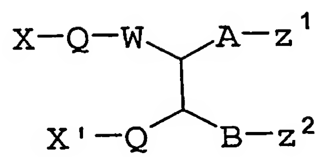
Claims

1. A compound of the general formula



(I)

in which one of X and X′ represents a polymer, and the other represents a hydrogen atom;

each Q independently represents a linking group;

W represents an electron-withdrawing moiety or a moiety preparable by reduction of an electron-withdrawing moiety; or, if X′ represents a polymer, X-Q-W- together may represent an electron withdrawing group; and in addition, if X represents a polymer, X′ and electron withdrawing group W together with the interjacent atoms may form a ring;

each of Z^1 and Z^2 independently represents a group derived from a biological molecule, each of which is linked to A and B via a nucleophilic moiety; or Z^1 and Z^2 together represent a single group derived from a biological molecule which is linked to A and B via two nucleophilic moieties;

A is a C_{1-5} alkylene or alkenylene chain; and

B is a bond or a C_{1-4} alkylene or alkenylene chain.

2. A compound as claimed in claim 1, in which a polymer X or X′ is a polyalkylene glycol, a polyvinylpyrrolidone, a polyacrylate, a polyoxazoline, a polyvinylalcohol, a

polyacrylamide or polymethacrylamide, a HPMA copolymer, a polyester, polyacetal, poly(ortho ester), polycarbonate, poly(imino carbonate), polyamide, a copolymers of divinylether-maleic anhydride or styrene-maleic anhydride, a polysaccharide, or polyglutamic acid.

3. A compound as claimed in claim 2, in which the polymer is a polyethylene glycol.

4. A compound as claimed in any one of claims 1 to 4, in which each linking group Q independently represents a direct bond, an alkylene group, or an optionally-substituted aryl or heteroaryl group, any of which may be terminated or interrupted by one or more oxygen atoms, sulphur atoms, -NR groups in which R represents an alkyl or aryl group, keto groups, -O-CO- groups and/or -CO-O- groups.

5. A compound as claimed in any one of claims 1 to 5, in which W represents a keto or aldehyde group CO, an ester group -O-CO- or a sulphone group $-SO_2-$, or a group obtained by reduction of such a group, or X-Q-W- together represent a cyano group.

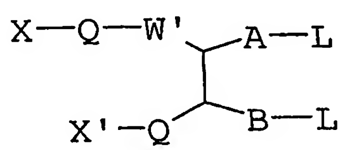
6. A compound as claimed in any one of claims 1 to 6, in which Z^1 and Z^2 together represent a single biological molecule

7. A compound as claimed in any one of claims 1 to 5, in which each of Z^1 and Z^2, or Z^1 and Z^2 together, represent a protein.

8. A compound as claimed in claim 7, in which the or each protein is linked to A and B via thiol groups.

9. A compound as claimed in claim 8, in which said thiol groups have been generated by partial reduction of a disulphide bridge.

10. A process for the preparation of a compound as claimed in any one of claims 1 to 9, which comprises reacting either (i) a compound of the general formula



(II)

in which one of X and X′ represents a polymer and the other represents a hydrogen atom;

Q represents a linking group;

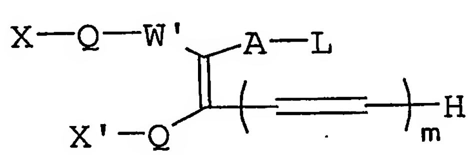
W′ represents an electron-withdrawing group, for example a keto group, an ester group -O-CO- or a sulphone group $-SO_2-$; or, if X′ represents a polymer, X-Q-W′ together may represent an electron withdrawing group;

A represents a C_{1-5} alkylene or alkenylene chain;

B represents a bond or a C_{1-4} alkylene or alkenylene chain; and

each L independently represents a leaving group;

or (ii) a compound of the general formula



(III)

in which X, X′, Q, W′, A and L have the meanings given for the general formula II, and in addition if X represents a polymer, X′ and electron-withdrawing group W′ together with the interjacent atoms may form a ring, and m represents an integer 1 to 4; with compounds of the general formula Z^1Nu or Z^2Nu or a compound of the formula $Z(Nu)_2$ in which Z represents a biological molecule, and each Nu independently represents a nucleophilic group.

11. A process as claimed in claim 10, in which the or each leaving group L represents $-SR$, $-SO_2R$, $-OSO_2R$, $-N^+R_3$, $-N^+HR_2$, $-N^+H_2R$, halogen, or $-O\emptyset$, in which R represents an alkyl or aryl group and Ø represents a substituted aryl group containing at least one electron withdrawing substituent.

12. A compound of the general formula II or III as defined in either claim 10 or claim 11.

13. A pharmaceutical composition comprising a physiologically tolerable compound as claimed in any one of claims 1 to 9, together with a pharmaceutically acceptable carrier.

14. A compound as claimed in any one of claims 1 to 9 for use as a medicament.

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